Application of Enzyme Bioluminescence in Ecology

Elena Esimbekova, Valentina Kratasyuk and Osamu Shimomura

Abstract This review examines the general principles of bioluminescent enzymatic toxicity bioassays and describes the applications of these methods and the implementation in commercial biosensors. Bioluminescent enzyme system technology (BEST) has been proposed in the bacterial coupled enzyme system, wherein NADH:FMN-oxidoreductase-luciferase substitutes for living organisms. BEST was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors for medical, environmental, and industrial applications. For widespread use of BEST, the multicomponent reagent "Enzymolum" has been developed, which contains the bacterial luciferase, NADH:FMN-oxidoreductase, and their substrates, co-immobilized in starch or gelatin gel. Enzymolum is the central part of Portable Laboratory for Toxicity Detection (PLTD), which consists of a biodetector module, a sampling module, a sample preparation module, and a reagent module. PLTD instantly signals chemical–biological hazards and allows us to detect a wide range of toxic substances. Enzymolum can be integrated as a biological module into the portable biodetector–biosensor originally constructed for personal use. Based on the example of Enzymolum and the algorithm for creating new enzyme biotests with tailored characteristics, a new approach was demonstrated in biotechnological design and construction. The examples of biotechnological design of various bioluminescent methods for ecological monitoring were provided. Possible applications of enzyme bioassays are seen in the examples for medical diagnostics, assessment of the effect of physical load on sportsmen, analysis of food additives, and in practical courses for higher educational institutions and schools. The advantages of enzymatic assays are their rapidity (the period of time required does not exceed 3–5 min), high

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sensitivity, simplicity and safety of procedure, and possibility of automation of ecological monitoring; the required luminometer is easily available.

Keywords Bioluminescence · Ecological monitoring · Enzymatic assay · Immobilization · Integral water toxicity · Luciferase

Abbreviations

ADH	Alcohol dehydrogenase
BEST	Bioluminescent enzyme system technology
FMN	Flavin mononucleotide
$FMN{\cdot}H_2$	Reduced flavin mononucleotide
L	Luciferase
L + R	Coupled enzyme system: NADH:FMN-oxidoreductase-luciferase
LI	Luciferase index
P	Induction period
PLTD	Portable laboratory for toxicity detection
R	NADH:FMN-oxidoreductase
RCHO	Long-chain aliphatic aldehyde
RCOOH	Corresponding fatty acid
TC	Toxicity coefficient

Contents

1 Introduction

Present-day analytical methods based on bioluminescent reactions catalyzed by various luciferases cover a wide range of analytical tasks from ecological monitoring of the environment (integral bioassays) to molecular diagnostics of various diseases and infections (high-sensitivity and specific bioluminescent immunoassays and heteroduplex analyses). The prospective viability of the application of bioluminescent methods is explained by their advantages:

- Broad range: Feasibility of constructing assays for a variety of processes and substances.
- High sensitivity: Possibility of detecting a target substance at concentrations down to 10^{-12} to 10^{-15} mol L^{-1}.
- Rapidity of measurement: Bioluminescent reaction lasts milliseconds, so usually the analysis time varies within the length of period from 1 to 30 min.
- Feasibility of constructing assays for individual specific molecules.
- The ability to test a number of key physiological and biochemical processes, for example, key metabolites such as NADH, flavins, glucose-6-phosphate, and so on; activities of key enzymes such as NAD^+ or NADH-dependent dehydrogenases and trypsin, among others; consumption of unique energy substrate such as ATP; intracellular dynamics of Ca^{++} (a universal regulator); ribosomal synthesis of protein molecules; genome structure and functioning; and others.
- Relative simplicity of methods.
- Availability of reagents and bioluminometers (devices for measuring light emission intensity).

The era of bioluminescent analytical studies began with the use of luminous bacteria enzymes, and continues to play an important role in bioluminescent analytics. Three factors determine the prospective viability of luminous bacteria and their enzymes in assays.

First, researchers had enough bacterial luciferase because it was possible to obtain large amounts of biomass; luminous bacteria had long been the only cultivated luminous organisms [1]. Recently, it has become possible to create recombinant organisms by introducing the *lux* genes into bacteria, which has solved the problem of getting luciferase from other luminous organisms for analytic purposes [2]. During those years when only bacterial luciferase was available, more than 100 methods were developed using luminous bacteria and their enzymes. Some of them are presented in the book series *Methods in Enzymology* published in 1978 and 1986 [3, 4] and in the reviews by Kratasyuk and Gitelson [5], Girotti et al. [6], and Roda et al. [7].

Second, bioluminescent assays using bacterial luciferase were characterized by high specificity of enzyme–substrate interactions.

Finally, it is known that the energy supply of bioluminescence occurs through the general metabolic chains of cells [8, 9]. This makes it possible to use the chains coupling enzymes with bacterial luciferase or build such chains artificially so that

the concentration of most key metabolites (and antimetabolites) or activities of key enzymes can be measured on the basis of luminescence.

At present, bacterial coupled enzyme systems NAD(P)H:FMN-oxidoreductase-luciferase are actively used in bioluminescent assays for ecology, medicine, agriculture, and other areas [7, 10]. In Reaction 1, luciferase catalyzes the oxidation of long-chain aliphatic aldehydes involving reduced flavin mononucleotide; one of the products of this reaction is a quantum of light in the blue-green spectrum (Reaction 1). To provide luciferase with reduced flavin mononucleotide, the luciferase reaction is coupled with the reaction catalyzed by NAD(P)H:FMN-oxidoreductase (Reaction 2).

$$\underset{\text{Luciferase } (L)}{\text{FMN} \cdot \text{H}_2 + \text{RCHO} + \text{O}_2 \rightarrow \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{h}\nu} \quad (1)$$

$$\underset{NAD(P)H : FMN-oxidoreductase\ (R)}{\text{NAD(P)H} + \text{FMN} + \text{H}^+ \rightarrow \text{NAD(P)}^+ + \text{FMN} \cdot \text{H}_2,} \quad (2)$$

Methods based on using enzyme–substrate systems from luminous bacteria can be nominally divided into two groups: selective analysis methods and integrated assays such as bioluminescent toxicity assays.

Despite the diversity of bioluminescent methods used in analytical studies, bacterial luminescence has long been the only bioluminescent system used for the assessment of chemical pollution of various objects or total toxicity of the test samples. Historically, the application of bacterial luminescence began with the use of luminous bacteria. Many works have been published describing the application of luminous bacteria for ecological monitoring [5, 6]. These methods made it possible to determine environmental pollution by comparing the luminous intensity of bacteria in the control and in the analyzed sample. As opposed to other test objects such as paramecia, algae, crustaceans, and so on, the bioluminescent bioassay was faster (the time of analysis didn't exceed 20 min); however, as with other living organisms, the essential disadvantage of this method was low accuracy of measurement caused by the "petulance" of live luminous bacteria: failure to maintain the stable state of bacterial culture during measurements and storage. The bacteria reacted to the appearance of toxic substances either by decreasing or by increasing the luminous intensity, which often led to ambiguous interpretation of results. That's why only qualified staff could work with bacteria. Because of these shortcomings a bioassay based on luminous bacteria didn't show very good results in ecological laboratories. To overcome those difficulties it was decided to use the enzymes of luminous bacteria both in soluble and immobilized states.

Since 1990, a new trend in bioluminescent analysis has been developing: bioluminescent enzyme system technology (BEST) [11], where the bacterial coupled enzyme system: NADH:FMN-oxidoreductase-luciferase substitutes for living organisms in bioluminescent enzymatic toxicity bioassays. In the presence of toxic agents, enzymes from luminous bacteria more closely reflect the toxicity of living organisms than do the use of chemical analysis and other current methods

that cannot solve the pressing problem of how to detect, identify, and measure the contents of the numerous chemical compounds that differ in their physicochemical and toxic characteristics. Moreover, BEST was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors for medical, environmental, and industrial applications.

The present work examines the general principles of bioluminescent enzymatic toxicity bioassays and describes the applications of these methods and their implementation for commercial biosensors.

2 Principles of Bioluminescent Enzymatic Bioassays

The main principle of bacterial luciferase-based analysis methods is detection of toxic properties in test substances and mixtures by their effect on bioluminescent enzymatic reactions [12–15].

Bioluminescent assays are based on the phenomenon of luciferase inhibition by the components of analyzed mixtures (Fig. 1). Many approaches and methods have been developed for design of in vitro bioluminescent assays, based on the bacterial coupled enzyme system: NADH:FMN-oxidoreductase-luciferase (L + R), especially for ecological monitoring.

2.1 The Bioassays Based on the Bacterial Coupled Enzyme System: NADH:FMN-Oxidoreductase-Luciferase

Application of enzymatic bioassays based on the bacterial coupled enzyme system NADH:FMN-oxidoreductase-luciferase for ecological monitoring instead of living organisms such as intact luminous bacteria, daphnia, algae, and other organisms that can be legally used in Russia is justified by the fact that NADH:FMN-oxidoreductase as part of enzymatic bioassays is present in all living organisms. That is why good correlation is expected between the effect of toxic substances on living organisms and on the coupled enzyme system of luminous bacteria. Integrated luciferase-based biotesting methods are mainly used for continuous rapid environmental monitoring in industrial and agricultural regions, for control over pollutant emissions of enterprises, for estimation of the efficiency of wastewater detoxification and of the control of water and wastewater treatment facilities, as well as for ecological risk assessment.

Bioluminescent integrated assays demonstrate changes in the bioluminescent signal as a reaction to the appearance of large-scale classes of polluting substances in the analyzed media. But the development of such assays depends on the influence of pollutants and xenobiotics on bioluminescence [16–18].

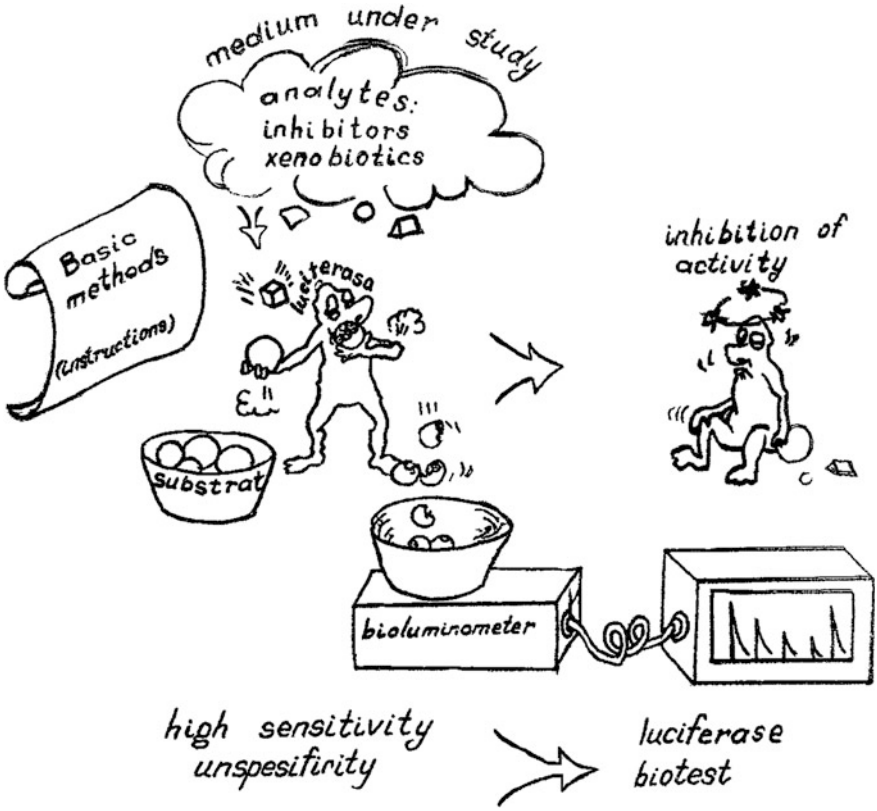


Fig. 1 Scheme of luciferase biotesting [11]

Enzymatic bioluminescent bioassays are based on the classification of bioluminescence inhibitors and activators according to the mechanism of their influence on fundamental physicochemical processes. The patterns of exogenous compound influence on bioluminescent systems are classified [19, 20]. According to this classification, there are four possible ways in which exogenous compounds act on a bioluminescent system: (1) influence of molecules on energy transport processes in a bioluminescent system, (2) influence of molecules on hydrogen transport processes in a bioluminescent system, (3) influence of molecules on electron transfer processes in a bioluminescent system, and (4) interaction of molecules with the enzymes of a bioluminescent system. Knowing the physicochemical basics of luminescent biotesting, it is possible to predict the analysis results and change the sensitivity of bioassays to certain pollutant groups [17, 18, 21–23].

When enzymatic biotesting is carried out, several analysis schemes can be used (Fig. 2). The first bioluminescent enzymatic bioassay variant is the quickest and demonstrates good repeatability of results. The following sequence of operations is performed: a cuvette with all the necessary components of a coupled enzyme

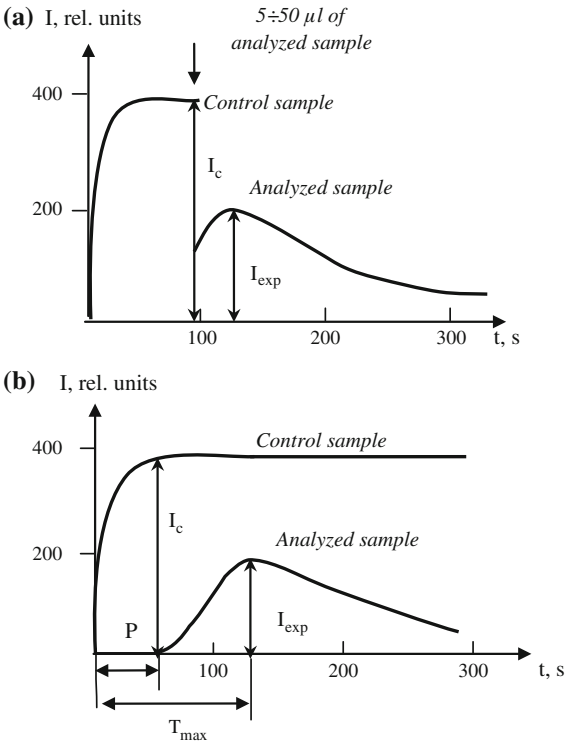


Fig. 2 Bioluminescent testing diagram (**a**); modified diagram of bioluminescent testing (**b**)

system of luminous bacteria, their substrates, and buffer solution are placed into a bioluminometer, and after the maximum light emission intensity I_c (control) is registered, from 5 to 50 μL of the test water sample or toxic substance solution are added, and the maximum light emission intensity I_{exp} is registered again (Fig. 2a).

The intensity of the residual luminescence $(I_{exp}/I_c) \cdot 100\ \%$ and the decay constant k_d are estimated. The decay constant is calculated according to the following formula: $k_d = [\ln(I_2/I_1)]/\Delta t$, where I_1 is the peak of bioluminescence intensity, I_2 is the bioluminescence intensity at the certain moment of time after reaching the bioluminescence maximum, and Δt is the time (minutes) needed for I_1 to reach I_2.

When analyzing toxicity of the water samples, the luciferase index (*LI*) or toxicity coefficient (*TC*) are calculated according to the formulas:

$$LI = \left(I_{exp}/I_c\right) \cdot 100\,\%,$$
$$TC = \left[\left(I_c - I_{exp}\right)/I_c\right] \cdot 100\,\%.$$

LI and *TC* are the residual luminescence and the degree of inhibition of the bacterial coupled enzyme system NADH:FMN-oxidoreductase-luciferase in the presence of analyzed sample correspondingly and $TC = 100 - LI$.

The criterion of toxicity is a 50 % decrease in the maximum luminescence level of the bacterial coupled enzyme system after the analyzed sample is added, as compared to the luminescence level in the control sample.

In the second case it is possible to achieve higher sensitivity of assays to the toxic substances. In this variant, testing of the control sample (usually distilled water or buffer solution) and analysis sample is performed in different cuvettes [24]. The reaction of the bioassay is also determined by the values of *TC* and k_d. But in that case, it is possible to use one more parameter for toxicity analysis: the time when the coupled enzyme system reached the luminescence maximum (T_{max}; Fig. 2b).

Application of the bacterial coupled enzyme system L + R for ecological monitoring is justified in quite a large number of works [18, 25, 26]. Bioluminescent bioassays based on the coupled enzyme system L + R were successfully used for the analysis of water in the Yenissei River and its tributaries, drinking water in various districts of Krasnoyarsk and Altai Territories, the salt lake Shira, as well as in Lake Baikal [27–29].

Bioassay reaction was studied in the analysis of potable and surface waters in the Altai region exposed to Semipalatinsk explosions and surface waters of the Yenissei River in the radioactive waste discharge area of the Mining-Chemical Combine (Krasnoyarsk) [26]. More than 100 samples of surface water and 170 water samples from wells and water supply systems have been analyzed. Results of the bioluminescent assay were compared with integral tests to assess surface water quality for its redox characteristics including hydrogen peroxide concentration, redox-status of aquatic medium, and OH-radical formation rate and lifetime. The assay makes possible to characterize water quality by three cleanliness classes according to PD 53.18.24.83-89 and define pollution (chemical or biological) causes [30]. In all parts of the Altai region the condition of surface and potable water caused concern. Results of the bioluminescent assay were confirmed by chemical and spectral analyses. In most samples results of bioluminescent assays have been found to correlate with the integral assay based on redox characteristics of the analyzed water [26].

Vetrova et al. [29] used bioluminescence bioassays based on luminous bacteria (*Photobacterium phosphopreum*) and adapted coupled enzyme system NADH:FMN-oxidoreductase-luciferase for monitoring the saline-water conditions of Lake Shira (Khakasia, Siberia). The saltwater Lake Shira has become a very popular health resort in southern Siberia, and the ecological problems of the lake are currently of great concern. The differences in bioluminescence responses have been found to be related to the salt composition and the oxidation–reduction properties of the water. Bioluminescent kinetics parameters, which are mostly sensitive to pollution under conditions of saline water, have been observed. The enzymatic system in the presence of 1,4-benzoquinone are shown to be more sensitive to redox characteristics of the salt water than in the absence of 1,4-benzoquinone. Thus, 1,4-benzoquinone should be included in a model solution for monitoring redox properties of the salt water. Using this technique, the results of bioluminescence analysis are used to construct a heterogeneity map that

characterizes the spatial and temporal water quality of Lake Shira. A partial map was based on the bioluminescence characteristics of water samples taken along the shoreline, sampling stations in the different places, and in different depths of the lake. The map reflects the correlation between the effect of the water samples on bioluminescence intensity and their chemical and physical–chemical characteristics. It has been demonstrated that the bioluminescence assay measurements must be done within 2 h after the sampling time.

The advantages of enzymatic assays based on the bacterial coupled enzyme system L + R are their rapidity (the time of analysis does not exceed 3–5 min), high sensitivity, simple measuring procedure, possibility of automation of ecological monitoring procedure, availability and safety of reagents, and a wide market of bioluminometers [31].

They were so easy to use and convenient that they started to be applied in the educational process, mainly in ecology practical courses [32–35].

2.2 Enzymatic Reagents for Bioluminescent Analysis

2.2.1 Ways to Obtain Stable Enzyme Preparations

Widespread use of the existing bioluminescent analytic methods is hampered by several disadvantages, including the instability of enzyme systems during use, limited shelf-life of enzymes–reagents for analysis, the need to control ambient conditions (i.e., pH, temperature, etc.) and interference by substances in the test samples, high manufacturing cost, and other factors [36].

These problems can be solved by using immobilized enzyme preparations in bioluminescent analysis, by obtaining reagents in the microenvironment that possess high catalytic activity and are stable for long-term storage, and making it possible to computerize analyses as biological modules of biosensors. Immobilization is the most effective and popular way among many known methods of enzyme stabilization. Advances in obtaining highly stable immobilized enzymes served as the basis for development of one of the foremost leading trends in biotechnology: creation of biosensors [37].

For the last 30 years immobilization has been widely used for production of stable reagents for bioluminescent analysis based on various bioluminescent systems: luminous bacteria, and bacterial and firefly luciferases. Many of the available immobilized reagents are successfully used in analytic measurements and in biosensors, because they simplify the analysis procedure, sometimes enabling full automation. At present, there are more than 40 different methods of immobilizing luminous organisms and enzymes isolated from various organisms [38].

The area of application of these methods is extremely wide: from analytical chemistry (analysis of NADH, FMN, ATP, and other substances), medicine (analysis of D- and L-lactate, bile acids in blood serum, amino acids such as alanine and phenylalanine, NADH-dependent enzymes, etc.), food industry (analysis of

the degree of bacterial content), ecological monitoring, and scientific research dealing with cell biology and in vivo enzymatic processes.

Production of immobilized reagents for analytic purposes is one of the major focal areas in applied enzymology. An important advantage of immobilized enzymes is the possibility to control the enzyme stability to physical and chemical environmental factors by way of choosing a suitable microenvironment. In an optimal microenvironment immobilized enzymes maintain high activity in a wide temperature range, and expansion of the range of pH optimum and ionic strength optimum is observed.

Table 1 shows the most effective methods of bioluminescent systems immobilization, and different immobilization methods are compared to reveal the advantages and disadvantages of each of them [38]. The activity of immobilized enzymes depends on the conditions under which the reagent was made. The optimal microenvironment for bacterial luciferase is natural polymer gel. To make a gel, gelatin or starch (potato, rice, or corn) can be used. By varying gel concentration, time, and mode of drying of immobilized enzymes it is possible to make reagents with different enzymatic activity [39–41].

The immobilized coupled enzyme system L + R does not require special storage conditions to save high enzymatic activity: when immobilized in starch or gelatin gel, it maintains its activity for 2 years [42]. Moreover, immobilization in starch and gelatin gel leads to a considerable stabilization of the coupled enzyme system with regard to denaturation treatment: pH optimum of the enzymes expands both to the acid and alkaline areas; high enzyme activity is maintained at increased salt concentration; thermal stability increases [43, 44]. The highest thermal stability is achieved in the coupled enzyme system immobilized in starch gel [40].

Apart from reagents including NADH:FMN-oxidoreductase and luciferase, there are reagents that include not only enzymes, but also the substrates of bacterial bioluminescent reaction. A patented stabilization and immobilization process preserves up to 50 % of the enzymatic activity and produces the homogeneous multicomponent reagent Enzymolum, which contains the bacterial luciferase, NADH:FMN-oxidoreductase and their substrates, coimmobilized in starch or gelatin gel [45]. The reagent is currently produced in tablet form and can be used in the cuvette variant of a bioluminometer (Fig. 3). The other forms (e.g. on the plane table, strips, and others) were also obtained for bioluminescent analysis. Enzymolum can be integrated as a biological module into the portable biodetector–biosensor of original construction.

Despite the significant advantages of immobilized reagents over soluble enzymes, the use of Enzymolum in toxicological studies was hardly possible at first, as the sensitivity of the bioluminescent coupled enzymatic system is not sufficient for testing pollutants at the maximum permissible concentration (MPC) level [17]. However, there are ways of increasing its sensitivity. This could be done by varying the conditions of testing, which is not possible for assays based on living organisms.

Table 1 Main features of different immobilization methods [38]

Polymer carrier	Relatively easy	Initial activity %[a]	Percentage activity at 4 °C after (days)[b]	Performance[c]	Turnover cycles	Automation
Polyacryl-hydrazide	Moderate 1–2	60	100 (15)	G—	20	Yes
BrCN-sepharose	Moderate 3–6	10–90	90 (5)	G 3–5	700	Yes
BrCN-agarose	Moderate 3–6	20–30	Stable (30)	VG 3–5	700	Yes
Epoxy-agarose	Moderate 3–6	0.1–1	70 (14)	P 0.5	–	No
Porous arylamino glass rods or beads	Moderate 6–10	1–2	Stable (15–180)	G—	700	Yes
Nylon tube reactor	Complex 6–8	50–70	100 (60)	G >1	900	Yes
Glass strips	Easy 1–2	92–96	100 (60)	G 1	100	Yes
Cellulose film	Easy 1–2	3–8	–	G 1	–	Yes
Lipid films	Complex 5–10	0.1–1	Stable (1–7)	P—	–	No
Bovine serum albumin gel	Easy <1	0,3–8	100 (30–60)	—1	100	No
Insoluble collagen	Easy 1–2	100	70 (14) 20 (240)[d]	G—	Several	Yes
Starch gel	Easy 10–12	100	100 (360)	VG 0.1	100	Yes
Alginate gel	Easy 2–10	30–60	100 (1)	G—	None	No
Polyamide membranes	Moderate 2–4	3–10	100 (180)	—1	None	No

[a] Relative activity compared with soluble enzyme
[b] Percentage of initial activity after number of days at 4 °C
[c] Reproducibility: *G* good; *VG* very good; *P* poor, or sensitivity relative to soluble enzyme set 1
[d] High mechanical strength

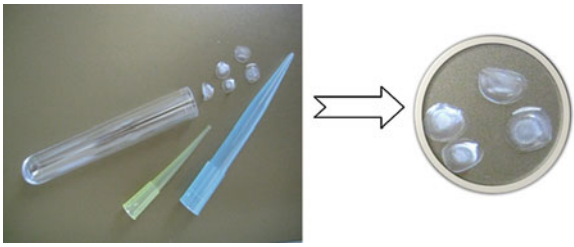


Fig. 3 The multicomponent reagent Enzymolum is a disk of dried film, diameter 6–7 mm; dry weight 1.5 ± 0.2 mg

2.2.2 Design of the Reagent Enzymolum and the Procedure of Toxicological Bioassay with Increased Sensitivity to Pollutants

The following methods were tested to increase the sensitivity of the reagent to pollutants: varying the composition of the immobilized reagent; varying the ratio of reaction mixture and test sample; varying the order of components added to the coupled enzymatic system and test sample; introducing an additional step of reagent incubation to the test water sample; and selecting the control solution for biotesting [46].

Varying the Composition of the Immobilized Reagent

Information about the dependence of bioluminescence kinetic parameters on the reaction mixture ratio, the concentration of enzymes and substrates, and enzyme stabilizers made it possible to increase the sensitivity of the bioassays. We studied the dependence of the sensitivity of NADH:FMN-oxidoreductase and luciferase immobilized in starch gel with the substrates of NADH and tetradecanal to the effect of benzoquinone and $CuSO_4$ upon the concentration of the immobilized reagent components. Figure 4 shows that the sensitivity of the multicomponent reagent increased as the enzyme concentration of its content decreased. A reagent with luciferase content 0.2 μg gave the greatest sensitivity: the residual luminescence intensity was 20–30 %. Changes in the other bioluminescence parameters (T_{max} and k_d), caused by varying the enzyme content in the multicomponent reagent, were insignificant. Increasing the NADH content in the reagent also decreased the multicomponent reagent's sensitivity to the effect of the analyzed pollutants. The maximum sensitivity to the effect of the analyzed substances was observed when the NADH content in the reagent was 0.1 mM (Fig. 4).

To prevent inactivation enzymes and to maintain their activity during use and storage, enzyme stabilizers with various mechanisms, including stabilizers of enzyme's SH-groups (DTT or mercaptoethanol) and BSA, were introduced to the multicomponent reagent. Introducing enzyme-stabilizing additives to the immobilized reagent increased the activity of the coupled enzymatic system L + R and the stability of immobilized enzymes during storage, but decreased the reagent's sensitivity to toxic substances (Fig. 5).

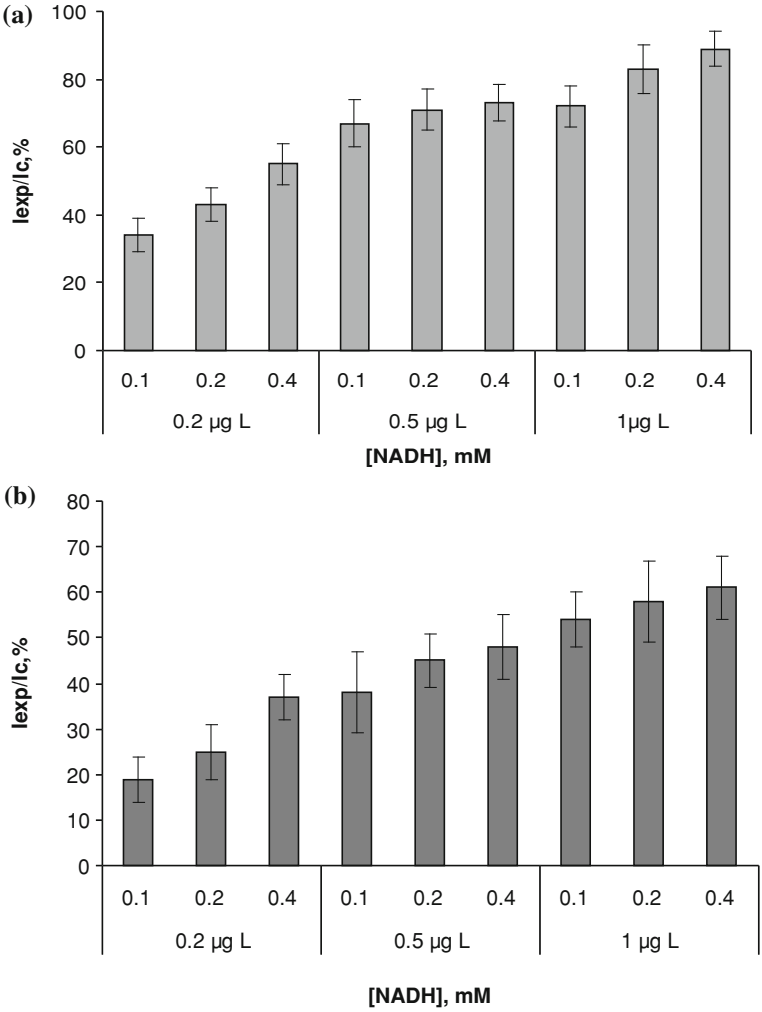


Fig. 4 Residual light intensity of immobilized reagent under variation of luciferase (*L*) and *NADH* content in reagent in the presence of 50 μg L^{-1} benzoquinone (**a**) or 5 μg L^{-1} $CuSO_4$ (**b**) [46]

It was shown previously [39] that introducing FMN into the immobilized reagent resulted in a significant decrease in the activity of the coupled enzymatic system; that is why FMN was not included in the reagent and FMN solution of various concentrations was added during the analysis. When the FMN concentration in the reaction mixture was increased, the sensitivity of the multicomponent reagent to benzoquinone decreased and remained at the same level to $CuSO_4$ (Fig. 6).

Thus, it is shown that by reducing the content of any component in the immobilized reagent, it is possible to increase the reagent's sensitivity to toxic

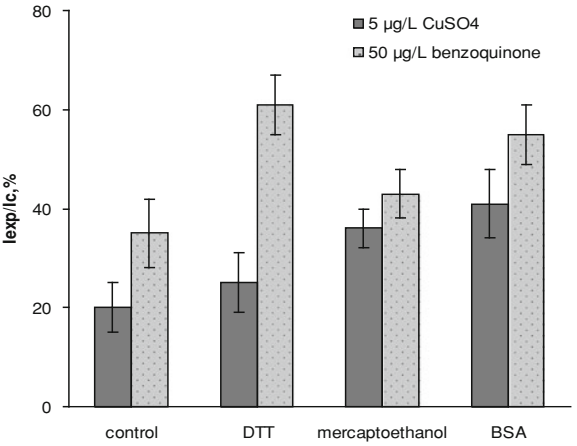


Fig. 5 Residual light intensity of immobilized reagent under variation of its content in the presence of 5 μg L^{-1} $CuSO_4$ or 50 μg L^{-1} benzoquinone. Content of stabilizers in disks was: *DTT* 0.1 mM, *mercaptoethanol* 0.2 mM, *BSA* 0.001 mM. *Control* is the reagent without any stabilizer [46]

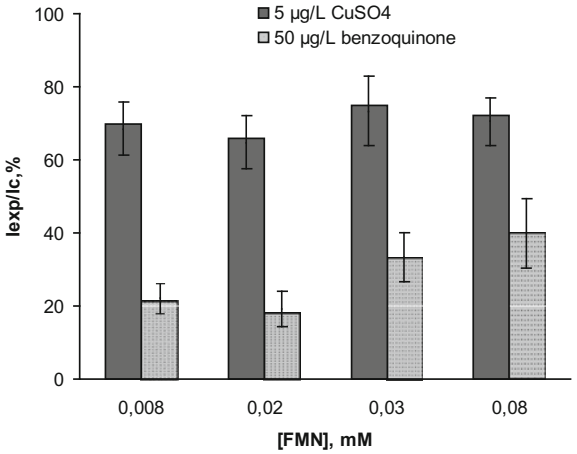


Fig. 6 Residual light intensity of immobilized reagent under variation of FMN concentration in the presence of 5 μg L^{-1} *$CuSO_4$* or 50 μg L^{-1} *benzoquinone*. *FMN* was injected as a solution [46]

substances. This effect can be explained by the fact that the coupled enzyme system had nonstationary conditions because the reagent contains a nonsaturating concentration of one of the substrates (NADH). Indeed, the maximum sensitivity of the coupled enzyme system to benzoquinone and $CuSO_4$ was observed when the NADH concentration was 1.3 times lower than the one corresponding to the Michaelis constant for this substrate [40].

Varying the Order of Components Added to the Reaction Mixture

In all the experiments described above, the pollutant solution was added to the reaction mixture after the maximum luminescence level had been reached, that is, after the substrates and enzyme active centers had interacted and, probably,

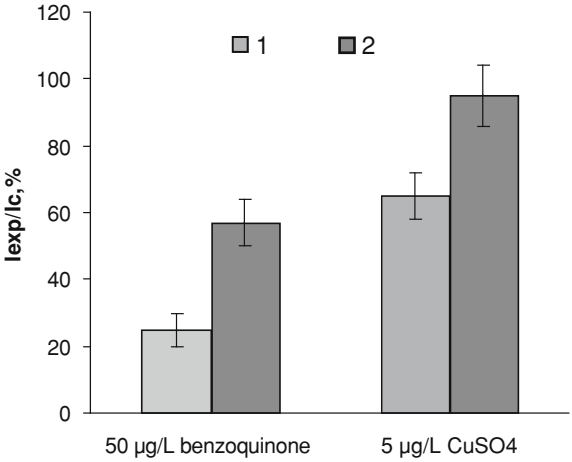


Fig. 7 Residual light intensity of immobilized reagent under variation of the order of introduction of the bioassay's components into the reaction mixture in the presence of $CuSO_4$ or benzoquinone: *1* introduction of analyzed water sample before starting enzyme reactions by FMN; *2* introduction of analyzed water sample after light intensity reaches its maximum [46]

formed enzyme–substrate complexes. The sensitivity of the coupled enzyme system can apparently be increased by injecting the analyzed sample before the maximum luminescence level has been reached, that is, before adding the last reaction substrate. In this case the increase in the reagent's sensitivity is explained by the enhancing of competition between the substrates and the added inhibitor toxicants.

Figure 7 shows that adding the analyzed sample before starting the reactions by the FMN solution led to an insignificant increase in the sensitivity of the immobilized coupled enzyme system to benzoquinone and $CuSO_4$.

Incubating Enzymes in the Test Solutions

The multicomponent reagent with the control or analyzed pollutant solution was incubated for 30–900 s, then the reaction was started with the FMN solution, and I_c and I_{exp} were measured (Fig. 8).

It was shown that such additional incubation increases the sensitivity of the immobilized reagent to copper sulfate, and the sensitivity to benzoquinone remains at the same level. The inhibition of enzymes with the copper sulfate solution grew proportionally to the increase of incubation time and reached its peak when the reagent had been incubated for 120–130 s with little effect on the assay sensitivity at 300-s incubation. A further increase of incubation time is impossible because the activity of the immobilized reagent is significantly lowered, even when the control solution is analyzed.

Thus, we found the conditions for biotesting aqueous solutions using the immobilized reagent that provide the maximum sensitivity to toxic substances.

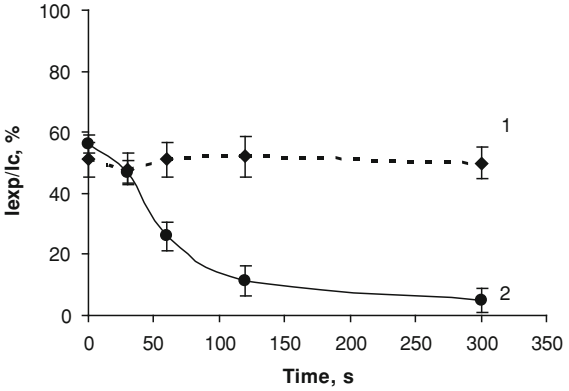


Fig. 8 Residual light intensity of immobilized reagent under variation of the time of reagent incubation in benzoquinone (*1*) or $CuSO_4$ (*2*) solutions [46]

Table 2 Effects of some pollutants on bioluminescence of soluble and immobilized coupled enzyme system NADH:FMN-oxidoreductase and luciferase [46]

Class	Substance	MPC, mg L^{-1}	EC_{50}, mg L^{-1}	
			Soluble L + R	Coimmobilized L + R, C14, NADH
Quinones	Benzoquinone	0.1	0.05	0.01
	Naphtoquinone	0.25	0.003	0.003
	Thymoquinone	*	0.005	0.03
	Toluquinone	*	0.01	0.05
Phenols	Hydroquinone	0.2	0.03	0.02
	Pyrocatechin	0.1	2.2	0.002
Heavy metal salts	$CuSO_4$	1	0.2	0.002
	$CdSO_4$	0.001	310	0.08
	CoC_{12}	0.1	40	0.1

*MPC isn't determined

Comparing Sensitivity of Soluble and Multicomponent Immobilized Coupled Enzyme System NADH:FMN-Oxidoreductase-Luciferase

The effect of pollutants upon the bioluminescent system was estimated according to the parameter EC_{50} which is the concentration of the active substance when bioluminescence is inhibited by 50 %. This parameter was chosen by analogy with the generally accepted parameter EC_{50} which is the effective concentration of the active substance causing a 50 % change in some vital functions of the test organism [47]. The values of EC_{50} were determined for various classes of organic pollutants using the soluble and immobilized coupled enzyme system L + R (Table 2).

Table 2 shows that the sensitivity of the soluble coupled enzyme system L + R to the studied pollutants was higher as compared to that of the immobilized

reagent. However, because the immobilized reagent enables measurements at the MPC level or even lower for almost all the studied substances, it can be used for biotesting natural and waste waters.

2.3 *Biotesting Natural and Waste Waters Using the Soluble and Immobilized Coupled Enzyme System NADH: FMN-Oxidoreductase-Luciferase*

The enzyme biotesting approach was used as a platform technology to certify "Method to measure the intensity of bioluminescence with the help of the 'Enzymolum' reagent to detect the toxicity of drinking, natural, waste and treated waste water" [24, 48]. The biotesting method of using an immobilized reagent was tested in analyzing the wastewaters of the pulp and paper plant. The characteristics of the samples are given in Table 3, where Sample #1 is the wastewater coming to the department, Sample #2 is the wastewater after mechanical treatment, and Sample #3 is the purified water from the outlet at the treatment facilities.

The criterion of toxicity was a 50 % decrease in the maximum luminescence level of the reagent after the analyzed water sample was added, as compared to the luminescence level in the control sample. The water's quality was determined by its toxicological characteristics, using the value of the biologically safe dilution, established by Russian Federation standards, according to Table 4.

The biotesting values were analyzed after samples had been diluted with distilled water or phosphate buffer. It is common practice in biotesting to use distilled water for diluting water samples, which makes it difficult to work with soluble enzymes. For example, there is a high level of luminescence inhibition by the control solution, distilled water, because of the discrepancy between the pH of distilled water (about 5.5) and the optimum pH of the coupled enzymatic system (about 7.0). Using 0.05 M potassium-phosphate buffer with pH 7.0 as the control solution can lead to a misinterpretation of biotesting results. If the environment studied is polluted with heavy metals, phosphates contained in the buffer cause them to precipitate, which reduces the effect of heavy metals on the luminescence of the coupled enzymatic system L + R. However, when soluble enzymes were replaced with an immobilized reagent that had a wider pH optimum range (from 5.0 to 9.0) than had the enzyme solutions [40], no such effect was observed.

Table 5 presents the results of wastewater biotesting using the immobilized reagent and under the Mann–Whitney U test, showing no difference between a sample diluted by water or buffer. According to the results, samples #1 and #2 were acknowledged to be hypertoxic, and sample #3 to be toxic. In addition, it was shown that the samples' toxicity decreases in a row: Sample #1 > Sample #2 > Sample #3, which corresponds to the changes in the chemical characteristics of the analyzed samples. This experiment confirmed the appropriateness and

Table 3 Results of the chemical analyses of waste and purified water samples

Component	Sample #1	Sample #2	Sample #3
pH	6.5	6.2	3.3
COD[a], mg L^{-1}	3,000	2,200	2,000
Dredge, mg L^{-1}	300	74	51
NH^+, mg L^{-1}	66	53	48
Nitrites, mg L^{-1}	<0.02	<0.02	<0.02
Nitrates, mg L^{-1}	6.2	0.1	0.1

Waste and purified water samples from the laboratory of the industrial waste purification department at the pulp and paper plant where sample #1 is the wastewater, sample #2 is the wastewater after mechanical treatment and sample #3 is the purified water [46]

[a] Chemical oxygen demand

Table 4 Toxicological characteristics of analyzed water in accordance with its dilution factor [46]

Dilution factor of analyzed water	Toxicological characteristics of analyzed water
1	Nontoxic
2	Slightly toxic
From 3 to 10	Toxic
From 11 to 50	Highly toxic

Table 5 Results of the toxicity bioassay of waste water at pulp and paper plant

Test water sample	Toxicity coefficient, %	
	Buffer[a]	dH_2O[b]
Sample #1	100	100
Sample #1, diluted 10 times	90.9 ± 7.9	90.5 ± 8.2
Sample #1, diluted 100 times	34.1 ± 6.6	31.0 ± 7.2
Sample #2	100	100
Sample #2, diluted 10 times	80.3 ± 8.0	79.4 ± 7.9
Sample #2, diluted 100 times	58.6 ± 7.4	56.6 ± 6.5
Sample #3	±10.2	86.5 ± 8.9
Sample #3, diluted 10 times	22.4 ± 4.8	18.7 ± 5.1

[a] 0.05 M potassium-phosphate buffer with pH 7.0 was used as a control sample and to prepare the analyzed water sample dilutions

[b] Distilled water was used as a control sample and to prepare the analyzed water sample dilutions

efficiency of using the immobilized reagent instead of the soluble coupled enzyme system L + R in biotests.

Another example is concerned with water bodies containing clean "reference" water, such as Lake Baikal. During the studies of water from Lake Baikal a comparison was made of assays based on a soluble and immobilized coupled enzyme system depending on the place and depth of sampling (Fig. 9). Samples from five control points were studied.

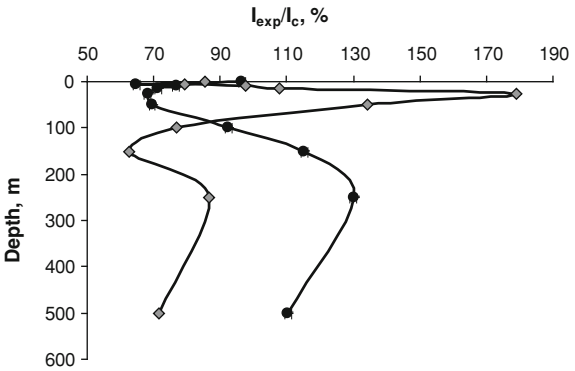


Fig. 9 Dependencies of the light emission intensity of a soluble (lyophilized) and immobilized couple enzyme system L + R on the depth of water sampling at Station 1 of Baikal lake

The nature of luminous intensity dependence for water samples from different depths was similar with both soluble and immobilized coupled enzyme systems. Water samples taken from the upper (the warmest) epilimnion zone didn't cause intense luminescence; it must be mentioned that, depending on the location of the sampling station, the zone was within the range of 5–50 m from the surface. Apparently, the slight stimulation effect could be explained by the fact that the epilimnion water, as compared to the other water body layers, contains more oxygen due to a larger phytoplankton biomass. Water samples from the depth of 50–100 m (depending on the place of sampling) had a slight effect on the luminous intensity with both soluble and immobilized coupled enzyme systems, which didn't suggest pollution, as the test readings were within the range of possible fluctuations of enzymatic assay parameters for "normal" unpolluted water.

2.4 The Reagent "Enzymolum" in Toxicological Bioassay of Air

Bioluminescent assays were used not only for aquatic environments, but also for monitoring of air pollution [49]. The sensitivity of luminous bacteria and their enzymes to air samples differing by industrial pollution degree was determined. Air samples were collected in the clean (Akademgorodok, sample #1) and polluted (the coal power plant area, sample #2) districts of the city of Krasnoyarsk. The air samples were collected into liquid absorption medium (water, ethanol, or acetone). The standard aspirating device performing 1.0 L/min was used. Chemical composition of the samples was analyzed with a gaseous chromatograph (Agilent Tech. 7890A). To compare the sensitivity of assays the numbers of dilution of the samples necessary to remove the toxic effect were considered (Table 6).

The results indicate that water is better than ethanol or acetone medium for air sample preparation because of its sufficient capacity to absorb organic compounds, and absence of interfering effects on bioluminescence. The sensitivity of soluble

Table 6 Comparative characteristics of bioluminescent assays used to monitor air pollution

Type of bioassay		Soluble coupled system	Luminous bacteria	Immobilized coupled system (Enzymolum)
Number of components (simplicity)		5	2	3
Duration of assay, min		10	5–30	7
Sensitivity (number of dilutions), sample#1/sample#2	Water	3/2000	3/700	3/16,000
	Ethanol	1/700	1/1	3/250
	Acetone	3/2,000	1/1	3/>2,000
Storage conditions		2 months at +15 °C, 3 years at –18 °C	6 months at +5 °C, 1 year at –18 °C	2 years at +4 to +25 °C

and immobilized enzymes is 3–24 times higher than the sensitivity of bacterial-based tests. The immobilized reagent provides the reduction of the time required to complete the analysis (down to 7 min), easy-to-use, higher sensitivity (allowed dilutions are up to 16,000), and the possibility to increase the volume of the sample up to 97 % of the total. Thus there is the possibility to apply the bioluminescent bioassays based on the immobilized reagent Enzymolum for air pollution monitoring [49].

2.5 *Signal System of Enzymatic Assays: Methodological Aspects*

In all the existing bioassays based on living organisms, the reaction of the test substance with the substances that are harmful for these objects is determined. In this context the term "environmental toxicity" is used, although it is not always correct. It is also considered that all living organisms, including humans, react with the test medium in a similar way. Such a conception is based on the statement that all living organisms have a similar structure and functions that are affected by toxic substances. The idea that normal vital activity of all living organisms—from amoebas to humans—is based on complex interconnected activity of many enzymatic systems providing with such functions as respiration, digestion, reproduction, bioluminescence, and the like, suggests that a universal test system could be developed consisting of a number of enzymatic reactions. Indeed, it is generally accepted that all living beings, despite the diversity of substrates involved in metabolism, including specific ones for a given organism, have similar basic types of reactions, and based on the activity of enzymes released under the effect of pollutants it is possible to determine the biochemical mechanisms of these substances affecting not only functions, but organisms on the whole.

Such a model enzymatic test system must include enzymes of different classes, or key enzymes of metabolic processes in living organisms. In this case it will be possible to determine which vital function of organisms will be inhibited by which toxic substance or their mixture.

Developing such a complex model test system is possible on the basis of bioluminescence. Using the coupling principle in bioluminescent analysis gives almost unlimited possibilities for determining the activity of various enzymes [5] and designing new enzymatic assays on this basis. The possible basis for development of new bioluminescent enzymatic bioassays is the coupled enzyme reaction NADH:FMN-oxidoreductase-luciferase.

The unique feature of the enzymatic analytical systems is that, due to the coupling with bacterial luciferase, it is possible to measure the activity of more than 100 enzymes [5] if enzyme coupling chains are created where the product of one enzyme is the substrate of the next one. The luciferase of luminous bacteria must be the terminal enzyme in coupling chains for such enzymes as lactic dehydrogenase, trypsin, glucose-6-phosphate dehydrogenase, and others, making it possible to measure the activity of many enzymes according to the luminous intensity.

Moreover, using enzymatic reactions instead of living organisms in toxicity bioassays makes it possible to regulate the sensitivity of enzymatic assays depending on the objective. For example, the sensitivity of enzymatic assays to the toxic substances may be increased by extending the coupling chain of enzymatic reactions [50].

As a result of using several chains of coupling enzymatic reactions with bacterial bioluminescence, a set of enzymatic assays sensitive to different groups of pollutants was created as a "signal system of enzymatic assays" [25, 51].

2.6 How to Design New Bioluminescent Enzymatic Bioassays

To develop new bioluminescent enzymatic assays, the following methods were suggested and used: extending the chain of enzyme coupling and using different enzyme interaction mechanisms and types. As an example, two enzymes were chosen: alcohol dehydrogenase (ADH) and trypsin [25]. These objects were chosen, firstly, because they belong to different classes (oxidoreductases and hydrolases), and secondly, because they interact differently with bacterial luciferase, providing different sensitivity to the effect of toxic substances [50, 52].

Next we are going to look at the principles of designing enzymatic bioluminescent assays in more detail. The easiest way to create an enzymatic bioassay is to use direct coupling of enzymatic reactions. Such a method of assay construction by making long enzyme coupling chains has been known for quite a long time. At first a bioassay was developed based on the monoenzymatic reaction of luminous

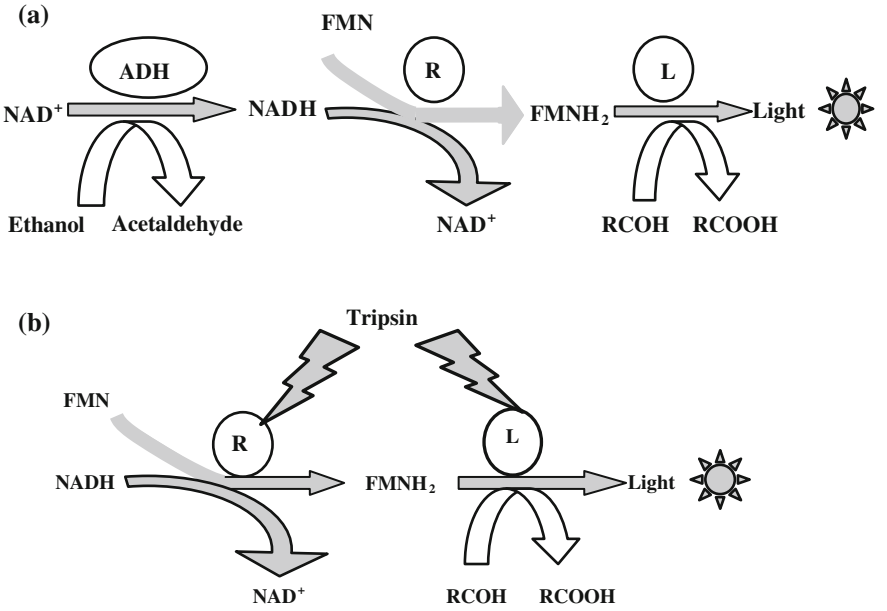


Fig. 10 Examples of coupling of the enzymatic reactions

bacteria (Reaction 1). Later a coupled enzymatic bioassay was created by coupling reaction (1) with NADH:FMN-oxidoreductase (Reaction 2) [11]. In the work of Kudryasheva et al. [50], three enzymes were coupled with direct ADH reaction as an example. In this case, NAD^+ was added to the reaction mixture that was subject to enzymatic reduction (Reaction 3) catalyzed by ADH, the resulting product NADH entered Reaction (2), and then other stages of the light emission process occurred.

$$C_2H_5OH + NAD^+ \overset{ADH}{\Leftrightarrow} NADH + H^+ + C_2H_4O \quad (3)$$

The luminous intensity during Reaction (1) is proportional to the concentration of $FMNH_2$ resulting from Reaction (2); the concentration of NADH depends on the activity of ADH in Reaction (3; Fig. 10a). It should be noted that bioluminescent methods used in enzyme activity measurement make it possible to couple more than three enzymes, thus enabling development of new enzymatic assays that can be applied for the analysis of toxicity of different samples.

Apart from direct coupling, there are other types of interaction between enzymes that can be used for the development of enzymatic bioassays. Using the example of reverse ADH-reaction (Reaction 3), a method was suggested for the creation of a triple enzymatic bioassay by adding a NADH-dependent reaction to the coupled enzyme system.

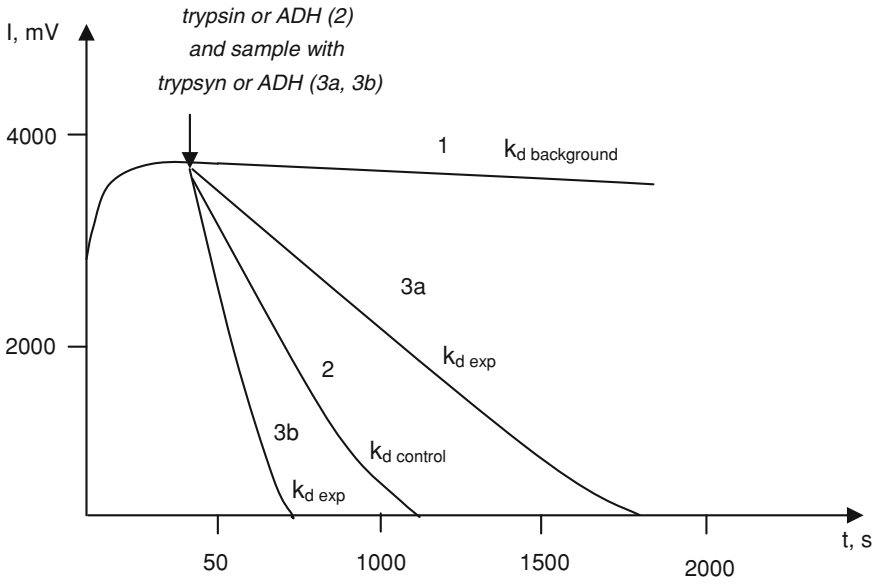


Fig. 11 Scheme of ADH (or trypsin) activity measurement using the bioluminescence decay constant: $k_{\text{d background}}$ is the decay constant for the coupled enzyme Reaction (*1*); $k_{\text{d control}}$ is the decay constant for the triple enzyme reaction with ADH or trypsin in the absence of pollutant (*2*); k_{dexp} is the decay constant for the triple enzyme reaction with ADH or trypsin in the presence of pollutant. *3a* the pollutant inhibits trypsin or ADH activity; *3b* the pollutant activates trypsin or ADH activity

The method determined the activity of NADH-dependent dehydrogenases, based on the measurement of the rate of reverse reaction catalyzed by dehydrogenase, which was developed by Petushkov et al. [53]. When a constant level of light is reached, acetic aldehyde and ADH are added to the coupled enzyme reaction L + R. Then, based on the quasi-first-order constant of bioluminescence recession proportional to the concentration of NADH, the activity of ADH is calculated (Fig. 11). This method is 20 times more sensitive than spectrometric measurements. The method is simple, one-stage, and the analysis time is 2–3 min. Similar methods have been developed to determine the activity of a number of other NADH-dependent dehydrogenases. All of them can be used as enzymatic bioassays.

The third way of designing enzymatic bioassays is based on the bioluminescent method used to measure the activity of proteolytic enzymes (Fig. 10b). It is based on the method of measuring the activity of proteases according to the bioluminescence recession constant [54]. When proteolytic enzymes are added to the reaction mixture, hydrolysis of luciferase and NADH:FMN-oxidoreductase occurs, causing a sharp decrease in luminous intensity (Fig. 11). Kudryasheva et al. [52] studied the effect of anthropogenic pollutants on proteolytic enzymes using trypsin as an example.

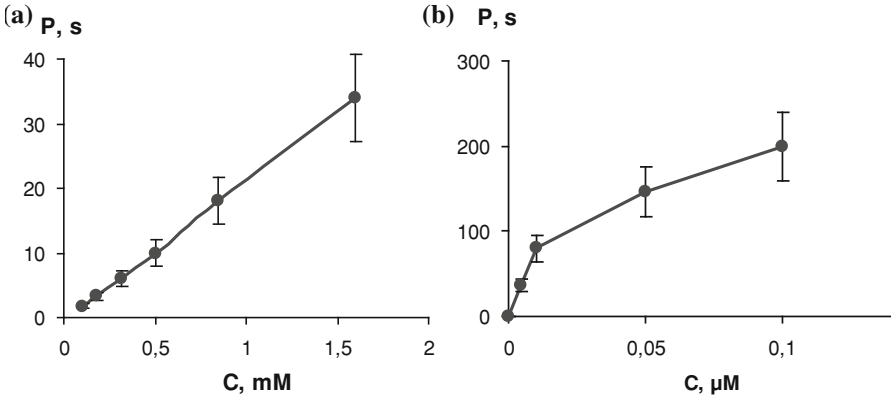


Fig. 12 Dependence of induction period on the concentration of 1,4-benzoquinone in the reaction mixture, **a** for the coupled enzyme system L + R, and **b** for the triple enzyme system ADH + L + R. Increasing the number of links in the coupling chain of enzymatic reactions results in a tenfold increase of sensitivity to benzoquinone [50]

Thus, estimation of the effect of chemical substances on the activity of the triple enzyme system with ADH and trypsin is based on changes in kinetic parameters of bioluminescence (Fig. 11). The procedure for triple enzyme biotesting is as follows.

- The maximum luminescence intensity of the coupled enzyme system L + R is measured, and then its luminescence intensity decrease is measured for 1 min. The decay constant ($k_{\text{d background}}$) for the case of the coupled enzyme reaction is calculated by the formula: $k_{\text{d background}} = \ln(I_{\max}/I_1)/(t_1 - t_{\max})$, where $I_{\max}$ is the bioluminescence intensity maximum and I_1 is the bioluminescence intensity at the moment of time t_1 (Fig. 11).
- 5–50-μL test sample and 5-μL ADH or trypsin solution are added to the reaction mixture described above. Then the decay constant $k_{\text{d exp}}$ is measured for the triple enzyme reaction.
- The authentic decay constant for the triple enzyme reaction is calculated by the equation: $k_{\text{d}} = k_{\text{d exp}} - k_{\text{d background}}$.
- For control measurement, 5-μL ADH or trypsin solution and 5–50-μL phosphate buffer (0.05 mol L^{-1}, pH 6.8) or distilled water are added to the reaction mixture described above. The control decay constant, $k_{\text{d control}}$, is calculated.
- Relative activity of ADH or trypsin is calculated using the equation: $A = (k_{\text{d}}/k_{\text{d control}}) \times 100$ %. The obtained value is proportional to the sample's toxicity.

Thus, three types of enzyme interaction were suggested for development of a set of enzymatic bioluminescent bioassays: direct reaction coupling, competition-for-substrate method, and proteolytic interactions. It was demonstrated that using different types of interactions it is possible to create enzymatic assays differing by their sensitivity to toxic substances. Kudryasheva et al. [50] showed that increasing

the number of links in the coupling chain of enzymatic reactions results in a tenfold increase of sensitivity to benzoquinone (Fig. 12).

The signal system of enzymatic bioassays was optimized and used for monitoring of natural and laboratory aquatic ecosystems and for studying the seasonal dynamics of zooplankton nonconsumptive mortality [55], as well as for toxicity analysis of pesticides [51] and sanitary assessment of natural polymers polyhydroxyalkanoates [56] and other applications.

2.7 Signal System of Enzymatic Methods for Toxicological Bioassay of Natural and Laboratory Aquatic Ecosystems

A signal system of bioluminescent assays was developed to monitor water quality in natural and laboratory ecosystems. It consisted of three enzymatic systems: coupled enzyme system NADH:FMN-oxidoreductase-luciferase and triple enzyme systems with alcohol dehydrogenase and trypsin. The results were compared with those for luminous bacteria.

The set of bioassays was applied to a small forest pond (Siberia, Russia), laboratory microecosystems polluted with benzoquinone and a batch culture of blue-green algae [25].

2.7.1 Dynamics of the Pond Ecosystem and the Microecosystem Components and the Bioassay Data

Prior to using these bioluminescent bioassays for ecological monitoring of water, it is necessary to answer the question: are the assays too sensitive; that is, do they respond to a natural variability of ecosystems, which is not connected to any pollution? That is why first the response of each bioassay to the water of the pond not subjected to heavy pollution was determined. The responses of the four bioassays for two extremely different parts of the ecosystem, epilimnion and hypolimnion, were compared [25].

The study was carried out at a small freshwater pond situated in a forest in the vicinity of Akademgorodok (Academic Town) of the city of Krasnoyarsk (Siberia, Russia). The pond has an oval shape, its surface area is about 3,000 m^2 and its maximum depth is 9 m. The samples were collected from epilimnion (depth about 2 m) and hypolimnion (depth about 7 ± 8 m) twice a week during three summer months.

Water temperature in the pond had ordinary summer variations (Fig. 13). A pronounced thermocline occurred at the depth of about 4 m in June–July and the temperature of hypolimnion (the layer below the thermocline) was 4 ± 10 °C lower than that of the epilimnion (the layer above the thermocline). In August the decrease of depth resulted in diminishing the difference between the epilimnion

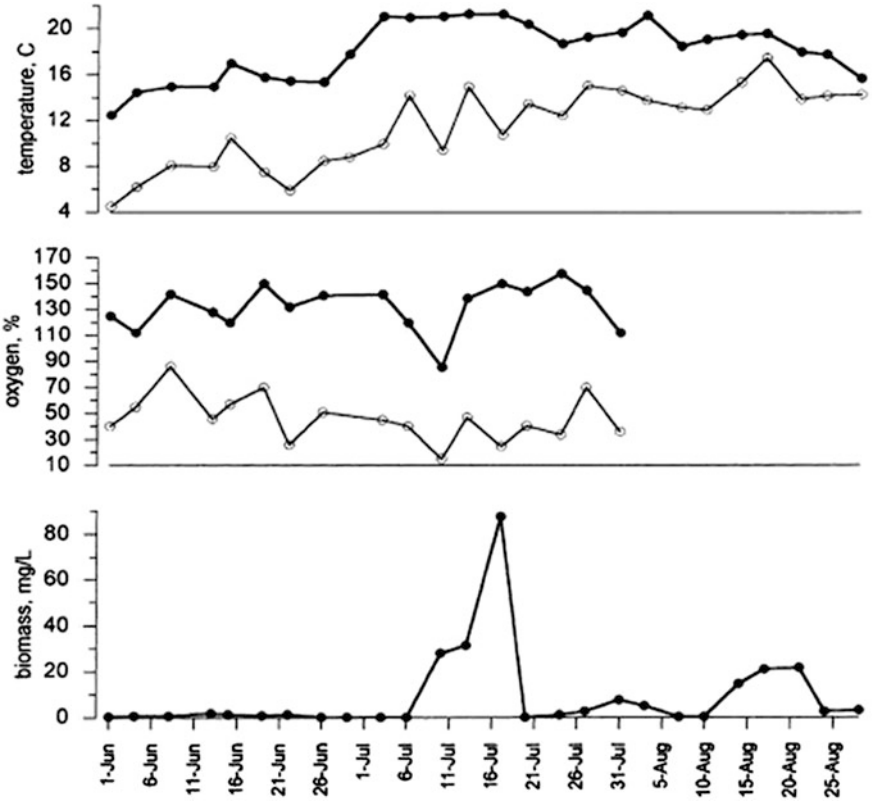


Fig. 13 Dynamics of the pond ecosystem components: (●) epilimnion, (○) hypolimnion [25]

and hypolimnion temperature. Dissolved oxygen saturation values (Fig. 13) in the epilimnion of the pond were significantly higher than those in the hypolimnion. In phytoplankton, green algae *Volvox aureus* Ehr. and diatom *Cyclotella comta* (Ehr.) Kutz. dominated. Blooms of *V. aureus* took place in the middle of July and in the middle of August (Fig. 13). *C. comta* dominated in the middle of June, when the water temperature was comparatively low. At the end of July and beginning of August, a small decrease of water temperature took place (Fig. 13) and *C. comta* became dominant in this period. In the hypolimnion only two samples of phytoplankton were taken in June and July, but the other dominant diatom and green species then in the epilimnion, *Stephanodiscus hantzschii* Grun., and *Closterium peracerozum* were found in the samples. Thus, the epilimnion and hypolimnion represented separate compartments of the pond ecosystems and naturally differed in water quality.

The variations of enzyme bioassay data were insignificant during the study period (Fig. 14). The data for the luminous bacteria based assay varied over the season. Water samples taken from the hypolimnion had a stronger inhibitory effect on bacterial luminescence.

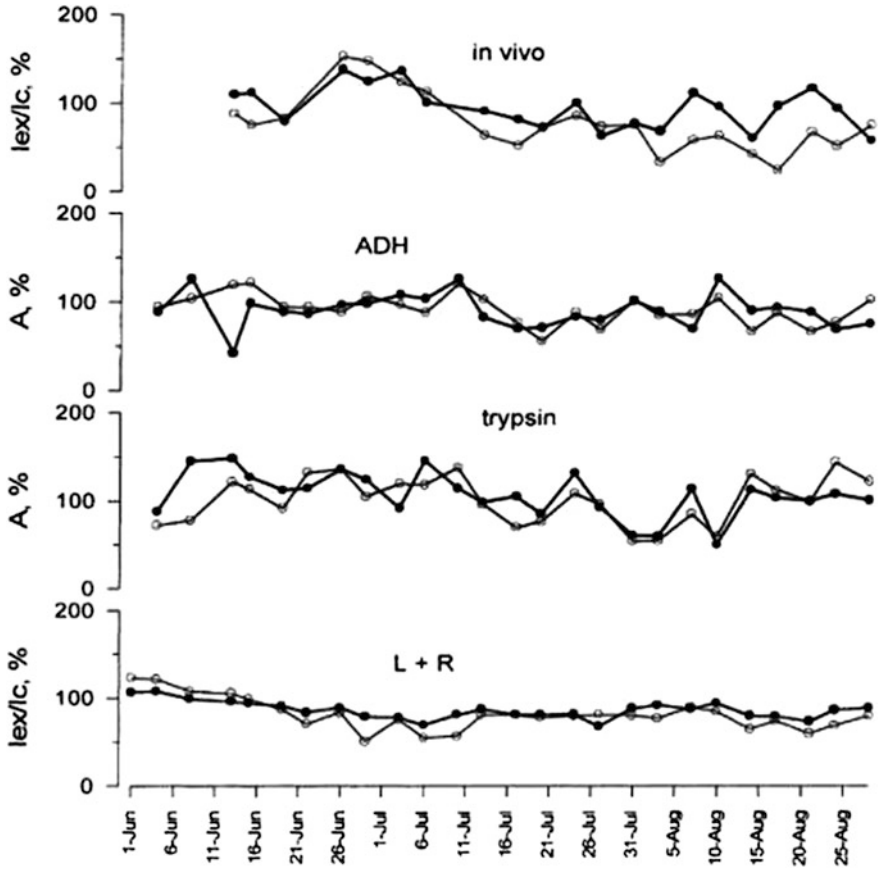


Fig. 14 Parameters of bioluminescent bioassays for the study of water from local pond: (●) epilimnion, (○) hypolimnion. A, %: relative activity of ADH and trypsin, I_c and I_{ex}: light intensity in control and experimental cuvette, respectively, for NADH:FMN-oxidoreductase-luciferase and luminous bacteria bioassays [25]

Only the bioassay based on the luminous bacteria demonstrated the significant difference between the epilimnion and hypolimnion by the Wilcoxon test. For the assays based on enzyme systems, the differences between the epilimnion and hypolimnion were insignificant.

At the end of summer water samples from the pond epilimnion (depth of about 2 ± 3 m) were inoculated into three cylindrical glass experimental microecosystems (MES) 25 cm in diameter and 25 cm high. They were illuminated by white light of 2.5 W/m^2 (natural intensity at the depth of sampling). Water samples from MES were taken twice a week for 2 months. Benzoquinone was added in the last week of study to MES 1.

The MES's experiment consists of two parts. In the first part (lasting 42 days; Fig. 15a) the relatively higher biomass of microalgae from the pond was

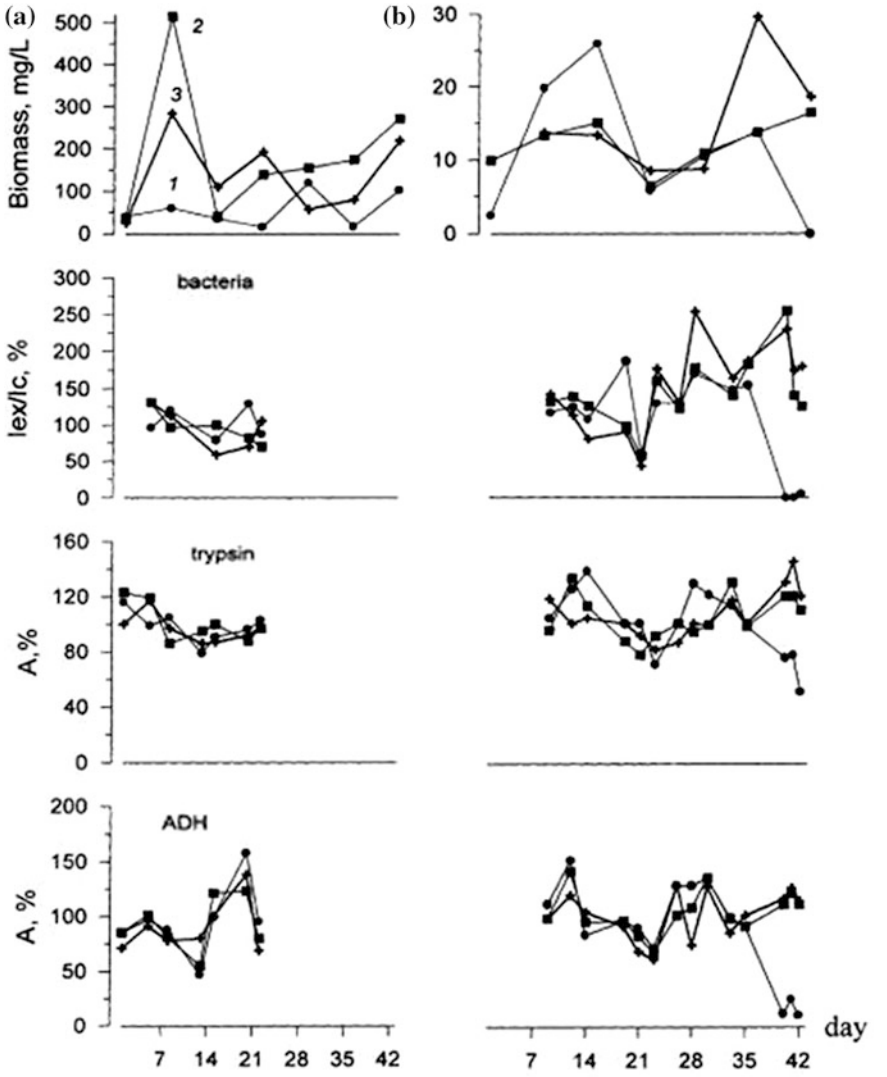


Fig. 15 Biomass of algae and the dynamics of bioassay parameters for MES: (●) MES 1 (treatment with benzoquinone on the 35th day of experiment (**b**), (■) MES 2, (+) MES 3. (**a**), %: relative activity of ADH and trypsin, I_c and I_{ex}: light intensity in control and experimental cuvette, respectively, for bacteria bioassay. **a** and **b** are experiments with high and low biomasses of microalgae, respectively [25]

inoculated into the MES 1–3 and the bioassay reactions at the high biomass were recorded. In the second part of the experiment (Fig. 15b) MES 1–3 were reinoculated and the low biomass of microalgae was maintained in MES 1 ± 3.

In the course of the experiment microalgal species composition in the MES were recorded as varying greatly. Throughout the experiments, green flamentous algae *Ulotrix tenerrima* Kutz., *Ulotrix variabilis* Kutz., *Spirogyra varyans* (Hass.) Kutz., and *Spirogyra tenuissima* (Hass.) Kutz., dominated in MES 2 and 3. In MES 1 during the first 17 days of the experiment A the dominating species was the flamentous diatom *Fragillaria virescens* Ralfs., then the green flamentous algae *U. variabilis* became dominant. Generally speaking, in all the MES the plankton community of diatoms and green microalgae of quite diverse species composition was replaced by a simpler community of flamentous algae.

Response of the bioassays ranged from inhibition of bacterial luminescence and ADH and trypsin relative activity to stimulation of parameters of these bioassays (Fig. 15a, b). Parameters of the bioassay based on the coupled enzyme system remained essentially unchanged throughout the investigations (Fig. 16). Besides, the response of this bioassay to water samples taken from different MESs was the same (Fig. 16). There was no correlation between the response of the bioassays and the total algae biomass.

After 35 days of experiment B, benzoquinone 10 mg $\times$ L^{-1} was added in MES 1. Benzoquinone was taken as a xenobiotic for two reasons. First, quinones are greatly responsible for the dissemination and destruction of the water stretches and their inhabitants [57]. Second, Kudryasheva et al. [58, 59] have shown that the bioluminescent system L + R is a specific assay for quinones. The concentration chosen was close to benzoquinone content in the wastewater of the paper industry and higher than LC_{50} for luminous bacteria *Photobacterium phosphoreum* 10 μg $\times$ L^{-1} [16]. When benzoquinone was added to MES 1, parameters of all bioassays changed drastically. The response of the bioassay based on the coupled system was a decrease of luminescence intensity, an increase in the time needed to reach the maximum, and the occurrence of the induction period on the first day of intoxication (Fig. 16). The luminous bacteria-based bioassay showed a complete absence of light ($I_{ex} = 0$) from the water from the microecosystem where the toxicant was added (Fig. 15b). The bioassay based on ADH and trypsin showed a decrease in the activity of the enzymes when the bioassay was performed on the sample where benzoquinone was added (Fig. 15b).

2.7.2 Bioluminescent Control for Blooming Water

"Bloom" in the water body can also change the response of the bioassays. It was shown earlier that bloom of blue-green algae was the characteristic feature of the ecosystem which indicated the distinctive rate and kinetics of the phenol biodegradation [60]. No bloom of blue-green algae was observed in the water body under investigation, thus we used a laboratory culture of blue-green microalgae *Spirulina platensis* as a model of a blooming pond.

Dynamics of bioassay data and growth of algae are depicted in Fig. 17. One can see that throughout the algal growth period, the response of the bioassay based on the coupled enzyme system varied insignificantly. It is evident from the assay data

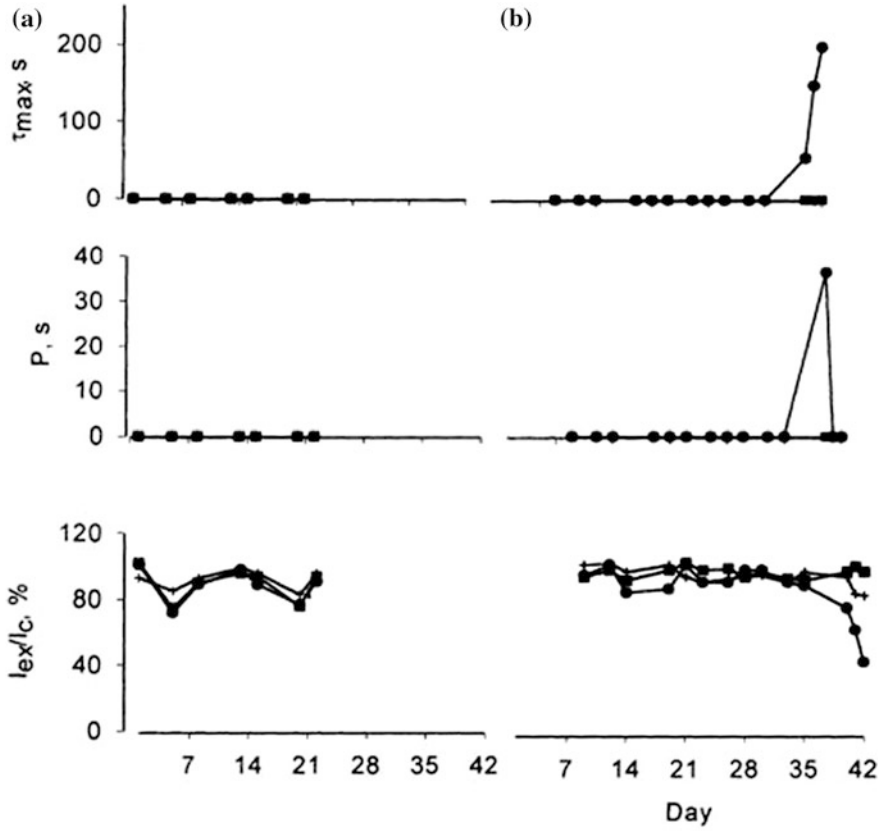


Fig. 16 Dynamics of parameters of bioassay based on coupled enzyme system NADH:FMN-oxidoreductase-luciferase for MES: (●) MES 1 (treatment with benzoquinone on the 35th day of experiment (**b**), (■) MES 2, (+) MES 3. **a** and **b** are experiments with high and low biomasses of microalgae, respectively, τ_{max} time of reaching the light maximum, P induction period, I_c and I_{ex} light intensity in control and experimental cuvette, respectively [25]

based on the triple enzyme systems that during the first days of their growth algae release metabolites inhibiting ADH and enhancing trypsin activity. After 4 days of algal growth, trypsin activity in the presence of the culture medium did not change. Response of the bioassays to the algal culture medium was the same in each of the three replicates. The bioassay based on ADH was the most sensitive. By the eighth and ninth days, the inhibition of the triple enzyme system with ADH was almost full (Fig. 17).

Therefore, bioluminescent assays show that their sensitivity differs by water quality, even in the case of the unpolluted pond. The assay based on luminous bacteria proved to be the most sensitive. The difference of water quality between the epilimnion and hypolimnion were due to natural causes rather than by any pollution. Hence, the result of this assay is determined by the depth at which water

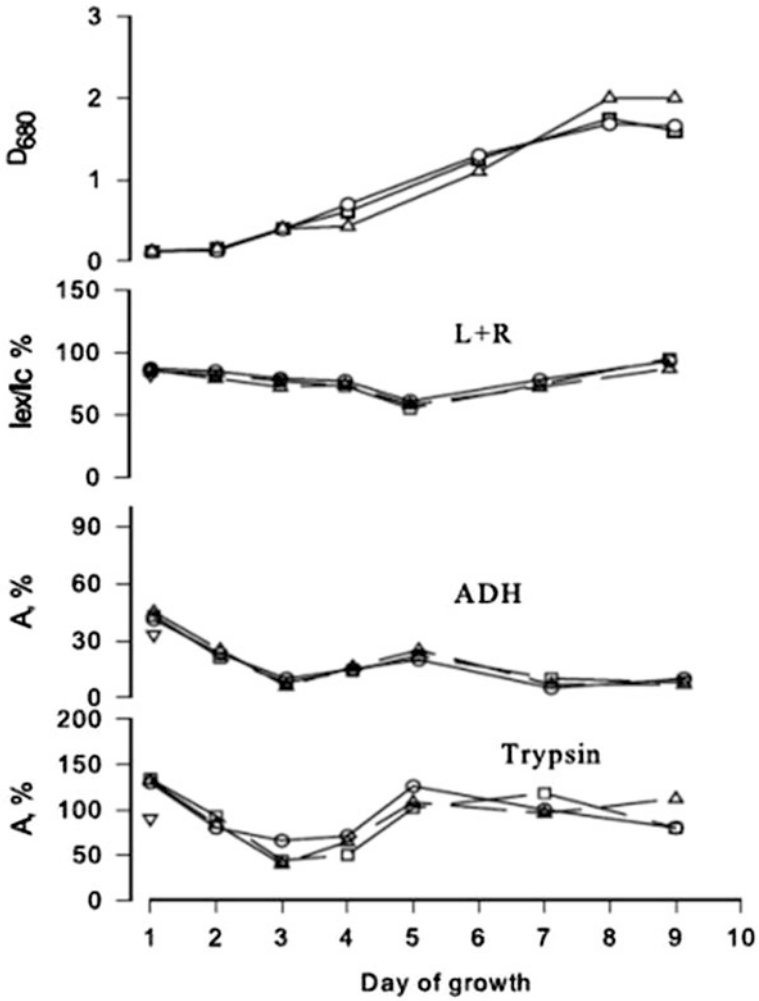


Fig. 17 The growth of microalgae biomass and the bioluminescent bioassay parameters for the study of the culture medium of *S. platensis*. (▽) effect of culture growth medium at the beginning of experiment, (○, □, Δ) three replicates, D_{680} optical density of *S. platensis* culture medium at 680 nm (the maximum of chlorophyll), *A* relative activity of ADH and trypsin, I_c and I_{ex} light intensity in control and experimental cuvette, respectively, for NADH:FMN-oxidoreductase-luciferase bioassay [25]

samples were taken (i.e., presence of the water from deoxygenated hypolimnion); this should be remembered when using this assay for the monitoring of water bodies.

Changes in the response of bioassays over the period of investigation were not related to pollution of the water body. Parameters of the bioassays did not change dramatically even in the periods of bloom of the green algae *V. aureus*. Indeed, in

contrast to blue-greens, bloom of green algae is not toxic. In addition, there is no correlation between a change in parameters of the bioassays and any of the pond ecosystem components (pH, dissolved oxygen, NO_2, NO_3, NH_4, etc.). From our data we could not determine the factor responsible for variations of parameters of the bioassays over the season. Apparently, such fluctuations in bioassay parameters should be regarded as natural for the unpolluted water body and must be accounted for by natural variability of water quality in the pond ecosystem.

It is evident from the experiments with the blue-green algae *S. platensis* that in the course of their growth these algae release substances that have the strongest effect on the ADH activity. Two components of the algal culture medium could change the response of the bioassays. They are culture medium mineral salts and metabolites released by algae as a result of their vital activity. Mineral salts are consumed by the growing algae, so their contribution to the inhibition of the enzyme activity decreases. The decrease of the ADH activity may take place due to an accumulation of metabolites in the medium at the end of the exponential growth phase.

There was no response of the coupled enzyme system, L + R, to the laboratory culture of the blue-green algae (Fig. 17). Meanwhile, the bioassay with trypsin responded to the algae medium at the early exponential stage of growth (the first 4 days). At this stage, the algae may release protease inhibitors into the culture medium.

Model experiments have shown that contamination of the MES with xenobiotics leads to sharp changes in parameters of all the bioassays. Here again, the most sensitive assay was that based on luminous bacteria. The most likely reason for this must be that xenobiotics (benzoquinone) affect a number of parameters of bacterial vital activity. They can affect respiratory and other oxidation-reduction processes in the cell, as well as disrupt membrane structures of the cell. As a result, the cells die, and do not produce the light.

The assay based on the coupled enzyme system is specific for quinones. If the water body is contaminated with quinones, a number of parameters of this assay change: P, T_{max}, intensity. The induction period appeared because the NADH was oxidized by benzoquinone. After the period of quinone reduction (P), by Reaction 2, FMN can be reduced, and further stages of the bioluminescent reaction with emission of a quantum of light become possible [59]. The ADH activity decrease may also be explained by the sequence of the redox NADH-dependent processes which may occur in the triple enzyme system NADH:FMN-oxidoreductase-luciferase-alcohol dehydrogenase [50].

The decrease of trypsin-relative activity may be explained by protein modification, for example, oxidation of the functional groups of amino acids contained in luciferase and trypsin (SH, etc.).

Thus, it has been shown that for the unpolluted water body fluctuations in the bioassay parameters were insignificant and resulted from natural variability of the pond ecosystem. Parameters of the assay changed sharply when the water body was contaminated with xenobiotics and in the case of bloom of blue-green algae. It is necessary to emphasize that ranges of variability of bioassays, which occurred in

the unpolluted pond and unpolluted MESs, were significantly lower than the degree of bioassay response after the addition of the pollutant (benzoquinone). Thereby we could detect the effect of pollutants such as quinones, within the variability of responses, caused by natural water.

Sensitivity of the assays to contaminants of polluted or "blooming" pond has been shown to differ, inasmuch as the mechanisms of xenobiotic (benzoquinone) action on the assay systems are different. Hence, the data of a single assay cannot provide a basis for a conclusion about the presence or absence of toxic substances in a water body. Only a set of assays, such as the one used in the present study can be applied as an alarm system to detect an acute toxicity of aquatic ecosystems.

2.8 *Signal System of Enzymatic Assays for Toxicological Bioassay of Pesticides*

Pesticides vary in their toxicity mechanism and character; for example, they can be carcinogenic or mutagenic, or they can affect the respiratory, endocrine, immune, or nervous systems [61, 62]. The effect of pesticides (organophosphates and pyrethroid preparations) on the bioluminescence of the four systems has been analyzed. The characteristics of the toxic substances analyzed are listed in Table 7.

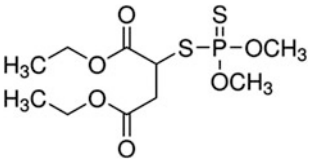
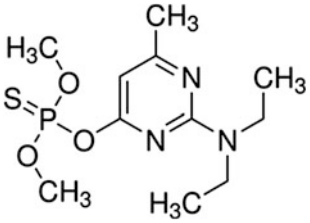
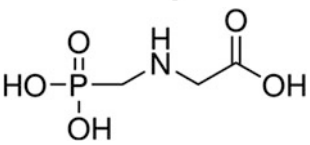
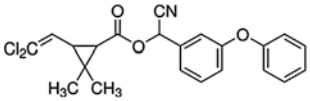
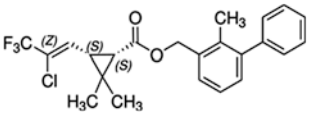
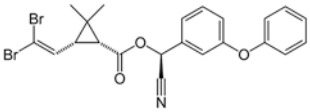
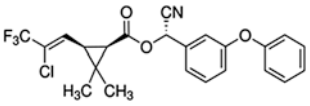
Four bioluminescence assay systems were used in our studies (Table 8). For each of the analyzed substances, values of EC_{50} and EC_{20} were obtained (Table 8). They constituted 50 and 20 % of the loss of luminescence for the coupled enzyme system and luminous bacteria, or 50 and 20 % loss of the relative enzymatic activity for the triple enzyme systems with ADH and trypsin. The parameter EC_{20} was taken for the authentic determination of a toxicant's impact on bioluminescence and for the comparison of the toxicant maximum permissible concentration.

The sensitivity of the coupled enzyme system to the impact of organophosphorous compounds differed widely. The EC_{20} of pirimiphos-methyl (Table 8, No. 2), for instance, was 0.9 mg $\times$ L^{-1}, whereas glyphosate (Table 8, No. 3) had no effect at all on the bioluminescence of the coupled enzyme system. It should be noted that glyphosate is a herbicide that has low toxicity to animals. Hence, their toxicity is in good correlation with bioluminescence inhibition.

The triple enzyme systems with ADH and trypsin are more sensitive to organophosphorous compounds (Table 8). The sensitivity of luminous bacteria to the impact of organophosphorous compounds is comparable to that of the coupled enzyme system (Table 8). For example, the EC_{20} of pirimiphos-methyl is the same in both cases, 0.9 mg $\times$ L^{-1}.

All the studied test systems were very sensitive to pyrethroid substances in the range 0.1–3 mg $\times$ L^{-1} (Table 7, Nos. 4–7). These insecticides, synthetic analogues of natural pyrethrins, act through intestinal contact, thereby affecting the nervous and the immune systems [63–65]. With pyrethroid, the values of EC_{20} are smaller for the bacterial system than for the coupled enzymatic system. To

Table 7 Characteristics of toxic substances analyzed by bioluminescence assays [51]

Number	Substance	Structural formula	Mr	Type of Substance
---	---		330.36	O
2.	Pirimiphos-methyl		305.33	O
3.	Glyphosate		169.07	O
4.	α-Cypermethrin		416.30	P
5.	Bifenthrin		422.87	P
6.	Deltamethrin		505.2	P
7.	λ-Cyhalothrin		449.85	P

compare: with deltamethrin (Table 8, No. 6) the values of EC_{20} are 0.06 and 0.001 mg × L^{-1} for the coupled enzyme system and luminous bacteria, respectively. This is attributed to permeability and destruction of cell membranes due to the high lipophilicity of these substances. Sensitivity of the triple enzyme systems to certain substances is similar to that of luminous bacteria.

Pyrethroid and organophosphorous compounds inhibited trypsin activity. Organophosphorous compounds are known to have an effect on proteolytic enzymes [61, 66]. They are able to inhibit esterase and proteolytic activity of both trypsin and chemotrypsin. This may account for the inhibitory effect of organophosphorous compounds observed in the triple enzyme system with trypsin.

Table 8 The influence of toxic substances on bioluminescent assay systems [51]

Number	Substance	Luminous bacteria photobacterium phosphoreum		Coupled enzyme system		Triple enzyme system with trypsin		Triple enzyme system with ADH	MPC (mg L^{-1})	PDD (mg kg^{-1})
		EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{20} (mg L^{-1})		
1.	Malathion	7	3.7	4	1.1	0.25	0.13	0.4	0.05	0.02
2.	Pirimiphos-methyl	5	0.9	3	0.9	7	3.3	11	0.01	0.01
3.	Glyphosate	40	30	Not measurable	>40	Not measurable	>40	>40	0.02	0.1
4.	α-Cypermethrin	0.5	0.31	0.7	0.3	1.61	0.16	10	0.002	0.01
5.	Bifenthrin	0.15	0.09	1.25	0.3	1.17	0.11	5	0.005	–
6.	Deltamethrin	0.01	0.001	0.5	0.06	1.3	0.32	0.003	0.006	0.01
7.	λ-Cyhalothrin	0.38	0.08	10	2.5	1.35	0.08	0.05	0.001	0.002

MPC maximum permissible concentration; *PDD* the person's permissible daily dose of a toxic substance (mg kg^{-1} body weight)

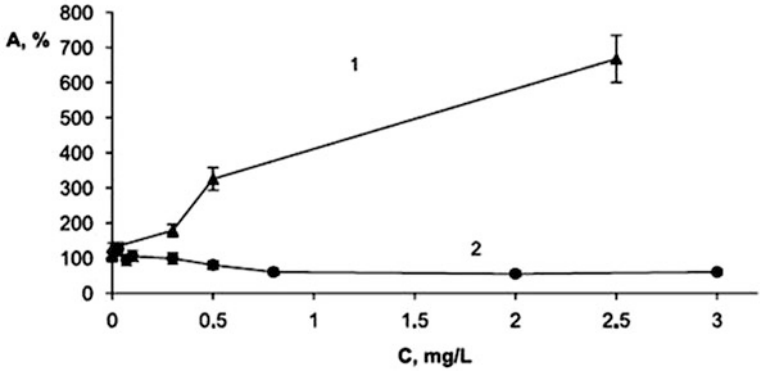


Fig. 18 Relative activity (*A*) of ADH (*1*) and trypsin (*2*) under different concentrations of deltamethrin. Influence of the pollutant on triple enzyme systems resulted in trypsin inhibition and ADH activation [51]

The triple enzyme bioluminescence systems included enzymes of different classes: ADH is an oxidoreductase and trypsin is a proteolytic enzyme. Therefore, the sensitivities and ways of interaction of ADH and trypsin with pollutants are different. All the toxic compounds studied, except malathion (Table 7, No. 1), had a weak stimulatory effect on the triple enzyme system with ADH. Figure 18 shows that deltamethrin inhibited the triple enzyme system with trypsin and activated the triple enzyme system with ADH.

Inhibition or activation of enzymatic activity by more than 20 % is indicative of the presence of toxic substances in the analyzed samples. Pesticides differ in their toxicity to animals [62, 65, 67, 68]. There are potent pesticides ($LD_{50} < 50$ mg $\times$ kg^{-1} animal's body weight) and pesticides of high (50–200 mg $\times$ kg^{-1}), medium (200–1,000 mg $\times$ kg^{-1}) and low (more than 1,000 mg $\times$ kg^{-1}) toxicity. Organophosphorous pesticides, with few exceptions, are of high and medium toxicity, but are rather quickly inactivated in the environment.

Table 8 gives the person's permissible daily dose (PDD) of a toxic substance (mg $\times$ kg^{-1} body weight) and the MPCs (the established hygienic standards of the Russian Federation: N. 1.2.1323-03). The values of EC_{20} for the studied bioluminescence systems are much higher than the MPCs. This means that the bioluminescence systems are not sensitive enough to detect a toxic substance at its MPC; however, it will allow the avoidance of a false signal given by background fluctuation of water quality, and allow authentic reaction with toxicants present in the water with concentrations exceeding MPCs, which are indeed dangerous for living organisms.

Considering EC_{20} and PDD (based on an average man's weight of 70 kg), these values are similar. By way of example, the PDD for malathion (Table 7, No. 1), corrected for a man's weight, is 1.4 mg $\times$ kg^{-1}, whereas the EC_{20} of the coupled enzyme system is 1.1 mg $\times$ L^{-1}. This shows that sensitivity of bioluminescence systems can be used to determine doses toxic to humans.

For pesticides, the sensitivities of the bioluminescence test systems were compared to those determined by other bioassays [62, 67, 69–72]. In some cases, bioluminescence bioassays are more sensitive. For example, the sensitivity of mice to deltamethrin (Table 7, No. 6), as established by the most sensitive (for mammals) hematological test, the erythrocyte micronuclei assay, is 90 mg $\times$ kg^{-1} [70], whereas the sensitivity of the bioluminescence test based on luminous bacteria is 0.001 mg $\times$ L^{-1}. The highest sensitivity reported was that of fish used in bioassays [62, 70]. However, analysis of that kind lacks fast response (the time required for testing is 7 days) and is very laborious.

Thus, bioluminescence tests are sufficiently sensitive to detect compounds toxic to humans and other mammals. The results presented can provide the basis for the development of an alarm test bioluminescence system based on either intact bacteria or any of several coupled enzyme systems. The described biotesting methods are proposed for measurements in the environment presumably contaminated with toxic agents as a result of industrial pollution.

Inasmuch as test systems differ in their sensitivities to various toxic agents, a deviation from the background in even one of the tests signals the presence of a "toxic factor" in the water. In this situation, chemical analysis is recommended.

3 Summary

It is necessity to point out that in vitro bioluminescent assay is a promising method for analyzing the integral characteristics of various media, and its potential hasn't been used up yet. For example, the coupled enzyme system is used for monitoring radiation toxicity, keeping track of the radiation effect on a microbiological and biochemical level. The bioluminescent method of radiation toxicity monitoring in solutions containing alpha- and beta-emitting radionuclides has been developed [73, 74].

A new trend in using bioluminescent methods is the efficiency assessment of detoxification by natural humic substances. This method is based on the quantitative determination of the antioxidant activity of natural detoxicants, humic substances. This method makes it possible to assess their remediation properties which are revealed by reducing the effect of toxic substances on the luminous intensity of bacteria bioluminescent reaction in the presence of humic acids [75, 76].

Moreover, the application area of bioluminescent enzymatic assays based on the estimation of integral toxicity is not limited by ecology. Methods based on the use of enzymatic bioluminescent bacterial systems are applied for food product quality. A method was suggested for detection of L- and D-lactate in beer [77]. The effects of the mycotoxins produced by fungi of the genus Fusarium on the coupled enzyme system: NADH:FMN-oxidoreductase-luciferase were studied [78–80]. A possibility was demonstrated to use bioluminescent methods for safety evaluation of food preservatives. Mei and coauthors [81] suggested detecting living bacterial

cells according to detection of cellular NADH using the coupled enzyme system: NADH:FMN-oxidoreductase-luciferase.

Integrated bioluminescent methods are also very promising for medical research, for example, for evaluating the gravity of endotoxicosis during treatment in surgery and therapy. The methods are highly sensitive, rapid, and simple and allow quantitative determination of the degree of seriousness of illness and the estimation of the severity of a patient's condition, disease course prediction, estimation of the efficiency of the used detoxification methods, and determination of the optimal operation mode of drainage facilities made of semipermeable membranes [82–84]. Controlled perfusion of rats' liver demonstrated a possibility of using an integrated bioluminescent method for the intensity assessment of pathologic oxidation processes [85]. A very interesting and promising trend in the development of integrated bioluminescent testing is the creation of rapid analysis for the assessment of human organism reaction to physical and mental stress. Analysis is made by comparing the light emission intensity of the coupled enzyme system NADH:FMN-oxidoreductase-luciferase in the presence of a person's saliva taken before and after a certain stress load [86]. The main advantages of the bioluminescent method are not only its rapidity, high precision, and sensitivity, but also noninvasiveness, because human saliva is analyzed, which reflects the functional state of a person just as blood does.

Thus, the new approach of biotechnological design developed on bioluminescent enzymatic biosensors, methods, and reagents has been described in the chapter. To solve the problem of how to detect, identify, and measure the contents of the numerous chemical compounds in environmental monitoring, food product monitoring, and medical diagnostics, we proposed Bioluminescent Enzyme System Technology BEST^{TM}, wherein the bacterial coupled enzyme system: NADH:FMN-oxidoreductase-luciferase substitutes for living organisms. BEST^{TM} was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors.

A patented stabilization and immobilization process produces the multicomponent reagent Enzymolum, which contains the bacterial luciferase, NADH:FMN-oxidoreductase, and their substrates, coimmobilized in starch or gelatin gel. The reagent is currently produced in tablet form and can be used only in the cuvette variant of a bioluminometer. The other forms, for example, on the plane table, strips, and others were also introduced for bioluminescent analysis. Enzymolum can be integrated as a biological module into the portable biodetector–biosensor of original construction.

Enzymolum is the central part of the Portable Laboratory for Toxicity Detection (PLTD), which consists of a biological module, a biodetector module, a sampling module, a sample preparation module, and a reagent module. PLTD allows us (a) to detect a wide range of toxic substances, more than 25,000 compounds; (b) to perform rapid screening for toxicity in emergency situations in the field and laboratories; (c) to develop systems for analyzing individual compounds; (d) to develop systems to evaluate the degree of overall toxicity; (e) to keep the high sensitivity of reagents for many years; and (f) to perform biotesting in the presence

of high concentrations of organic substances in water. The last item suggests the uniqueness of enzymatic bioassays, because no bioassays based on living organisms give consistent results under such conditions. Living organisms use organic substances for food and thus bioassays based on living organisms do not reflect environmental quality correctly, resulting in the absence of accuracy and reliability of analysis.

Prototype biosensors developed with this technology offer cost advantages, versatility, high sensitivity (up to 10^{-14} mol of analyte), rapid response time (less than 3 min), extended shelf life (up to 2 year), and flexible storage conditions (up to +25 °C).

The enzyme biotesting approach was used as a platform technology to certify "Method to measure the intensity of bioluminescence with the help of the 'Enzymolum' reagent to detect the toxicity of drinking, natural, waste, and treated waste water" [48]. The laboratory will be the principal example of a whole family of new, portable, professional laboratories for ecological monitoring, food quality laboratories, military departments, and other monitoring, teaching, security, and research organizations.

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